Evidence for an Unidentified Growth Factor(s) from Alfalfa and Other Plant Sources for Young Guinea Pigs [1,2]

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ABSTRACT An improved purified diet for the guinea pig has been described which is suitable for assay of a proposed unidentified growth factor(s) found in alfalfa and other plant sources (such as soybean meal, dried broccoli, and dried grass clippings). Improvement in growth of up to 50 g over a 3-week assay period was obtained by adding 10% of dehydrated alfalfa to the diet. The factor(s) appears to be distinct from all known vitamins, minerals, amino acids, or other nutrients. It appears to be organic in nature, since it is not found in the ash of alfalfa. The relationship of this "plant factor" to the "grass juice factor" or to other reported unidentified factors for other animals is unknown at this stage. Attempts to identify the factor(s) are in progress.

Although it is possible today to rear and reproduce experimental animals with diets consisting of synthetic or highly purified ingredients, reports are still being published (chiefly of studies utilizing the fowl) of unidentified growth factors present in a variety of natural ingredients such as fish meal, condensed fish solubles, meat meal, distillers solubles, green plants, and alfalfa.

Shortly after the development of an improved semipurified diet for young guinea pigs in 1953 (1), an improved commercial stock diet [3] became available which resulted in 10 to 15% greater early growth than that obtained with a semipurified diet.[4] It soon became evident, in experiments reported here, that this difference in growth could be overcome largely by adding either 20% of the stock diet or a wide variety of plant materials to the semipurified diet. Dried alfalfa was found to be one of the better sources of the growth factor(s).

Alfalfa has been reported to contain an unidentified factor(s) for poultry (2–6), for a microorganism (7), and for the pig (8). In 1938, Kohler et al. (9) studied the growth effect of the "grass juice factor" in guinea pigs, although this factor has since been shown to be largely replaced by a variety of known essential nutrients (10), and its status is uncertain. In 1957, Ershoff (11) reported that both dried alfalfa juice and water-washed alfalfa pulp had significant growth-promoting activity for guinea pigs fed a "mineralized spray-process dried milk" ration (with unstated, and uncertain, amounts of all nutrients now known to be required by the guinea pig). Supplementation of alfalfa meal reduced mortality in guinea pigs exposed to irradiation in studies made by Calloway et al. (12). Reid and Mickelsen (13) confirmed our earlier report [5] that dried alfalfa increased the growth of guinea pigs when added to semipurified diets very similar to those used here.

The purpose of this study is to provide information on various crude sources of the unidentified growth factor(s), on an

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[2] A preliminary report of part of this study was presented at the American Institute of Nutrition meeting in April, 1961 (Federation Proc., *20*: 236, 1961). Details of these and related experiments may be found in the M.S. theses of Jean Reiche Davis, 1961, and Raj K. Lakhanpal, 1963, deposited with the Graduate Division of the University of California, Berkeley.

[3] Purina Guinea Pig Chow, Ralston Purina Company, St. Louis.

[4] Unpublished data, G. M. Briggs.

[5] See footnote 2.

improved assay, on some minor attempts of preliminary fractionation, and on differentiation of the factor(s) from known nutrients.

EXPERIMENTAL

Animals and their care. More than the usual details of animal care are given here because of their importance to the success of the experiments and because of the sensitivity of the very young guinea pig to environmental changes. Guinea pigs of mixed strains and mixed sexes, 6 to 9 days old and weighing 95 to 120 g, were obtained commercially.[6] For the first 2 or 3 days, before the start of the experiments, groups of 4 to 6 animals were maintained with the basal diet in double-size cages in order to facilitate learning the use of the drinking apparatus and feed cups. The animals were then divided into uniform groups of five or more according to sex and weight, placed individually in single cages, and fed the various diets. The metal cages (24 × 18 × 18 cm) had 0.6-cm mesh floors the first week, and 1.3-cm mesh floors thereafter. Shallow china food cups (30 mm high and 70 mm in diameter) were used; each cup had a metal top with a large round hole (45-mm diameter) in the center to keep food contamination by the young guinea pigs at a minimum. After the first week, larger cups (60 mm high and 65 mm in diameter) without metal tops were used. Each feed cup was kept in a small wire-mesh frame attached to the cages to prevent tipping. Tap water was supplied from inverted bottles with copper or stainless steel drinking tubes.

The young guinea pigs were handled gently and special attention was given to see that they learned to eat and drink readily. They were checked several times a day for the first few days to see that food and water supplies were clean, available, and being consumed. Cages were cleaned and sterilized 3 times per week or more often, as needed. The animals were weighed daily for the first 3 days and 3 times per week for the rest of the experimental period. Their food consumption was recorded and estimates for food wastage and spillage were made daily.

Most of the earlier feeding trials with young guinea pigs were of 2 weeks' duration. Because the latter phase of the study utilized animals from new sources and these were highly susceptible to infections and environmental changes during the early period, the feeding trials were changed to a 3-week period. Under these conditions, when an occasional animal died during the first week (an average of about 1 in 10 animals, but in some experiments as many as 1 out of 4), it was replaced with an extra animal of the same sex and of similar weight that had been fed the basal diet. Such deaths were obviously due either to an infection or to a refusal to eat or drink and not to the effects of the nutritive value of the diet. Few animals died after the first week, as shown in the tables.

Experimental diets. The composition of diets GPD2 and GPD3 is shown in table 1. Diet GPD2 is a modification of diet

TABLE 1

Composition of basal diets

	Diet GPD2	Diet GPD3
	g/kg	*g/kg*
Casein (vitamin-free)	300	300
Cornstarch	200	—
Glucose hydrate [1]	64	64
Sucrose	100	462
Cellulose [2]	150	—
Corn oil	50	50
Mineral mixture (HMW) [3]	60	50
Potassium acetate	25	25
Magnesium oxide	5	5
Zinc carbonate	0.13	0.13
Ascorbic acid	2	2
Choline chloride	2	2
Inositol	2	—
B-vitamin mix in glucose [4]	20	20
Fat-soluble vitamin mix in corn oil [5]	20	20
Total	1000.13	1000.13

[1] Cerelose, Corn Products Company, San Francisco.
[2] Cellophane Spangles, Rayon Processing Company, Pawtucket, Rhode Island.
[3] Hubbell, R. B., L. B. Mendel and A. J. Wakeman. J. Nutrition, *14:* 273, 1937; obtained from Nutritional Biochemicals Corporation, Cleveland.
[4] The following amounts were present per 20 g glucose: (in mg) thiamine·HCl, 16; riboflavin, 16; pyridoxine·HCl, 16; Ca pantothenate, 40; niacin, 200; biotin, 0.5; folic acid, 10; and vitamin B_{12}, 0.05.
[5] The following were present per 20 g of corn oil: (in mg) vitamin A acetate, 6; α-tocopheryl acetate, 20; vitamin D_3, 0.04; vitamin K (menadione), 2.

[6] Supplied by Dependable Animal Supply Company, Martinez, California. In the early studies, guinea pigs from the N.I.H. colony were used.

GP13 of Reid and Briggs (1). Diet GPD3, a further modification, contains no added source of inositol, cellulose, nor starch. These deletions were made in an attempt to remove possible contaminating sources of sparing factor(s) or the unidentified factor(s) itself. Diet GPD3 also contained 1% less mineral mixture to avoid slightly detrimental effects with the larger amounts in this ration. Supplements were added to the diets at the expense of starch and sucrose, respectively. Fresh diets were mixed [7] every 2 weeks and stored in refrigerators when not in use. Commercial pelleted diets [8] for guinea pigs were used as a control diet (stock diet).

Fractionation of alfalfa. Weighed quantities of dehydrated alfalfa (commercial alfalfa meal, 17% protein) were extracted with 95% ethyl alcohol for 48 hours in a large Soxhlet apparatus. The alcohol extract was concentrated under vacuum in glass to a thick syrup which was carefully collected and weighed. The residue left in the thimble was dried at room temperature. The water-soluble "alfalfa juice concentrate" was prepared according to the method of Kohler and Graham (2).[9] The "dry" ash of alfalfa was prepared in a muffle furnace at slowly increasing temperatures to permit slow charring yet to allow removal of organic material within a 24-hour period. These fractions were fed at levels equivalent to 10% of the original dehydrated alfalfa meal.

A variety of substances, including alfalfa, as well as certain pure components, were tested either singularly or in various combinations, at levels indicated later, for their growth-promoting activity.

RESULTS

The effect of adding different levels of alfalfa to diet GPD2 in the early phases of this work is summarized in table 2, part 1. Optimal growth increases were obtained in 2-week studies with a minimum of 10% of alfalfa, the level chosen for use in later experiments.

In later 3-week studies (table 2, part 2), significant growth responses were again obtained with the addition of 10% dried alfalfa to the diet. The addition of

[7] Details of mixing the diets may be found in the theses mentioned in footnote 2.
[8] See footnote 3.
[9] Kindly supplied by Dr. George Kohler, Western Regional Research Laboratory, Albany, California.

TABLE 2

Supplementation of diet GPD2 with various levels of alfalfa meal

Supplement to diet GPD2 [1]	No. of experiments	No. of guinea pigs	Gain	Gain over basal
			g	*g*
Part 1. Experiments conducted 1960–62, 2-week tests [2]				
None (basal)	28	140	61	—
5% alfalfa meal (17%)	2	10	70	9
10% alfalfa meal	9	45	92	31
20% alfalfa meal	2	10	80	19
40% alfalfa meal	1	5	69	8
10% stock diet [3]	3	14	87	26
100% stock diet [3]	9	54	98	37
Part 2. Experiments conducted 1962–63, 3-week tests [4]				
None	3	17(17)	104	—
10% alfalfa meal	5	31(30)	135	31
Alcohol extract of alfalfa (equivalent to 10% alfalfa)	3	19(19)	110	6
Alcohol residue of alfalfa (equivalent to 10% alfalfa)	4	25(25)	112	8
10% dried grass clippings	1	6(6)	136	32
100% stock diet [3]	2	9(8)	140	36

[1] Supplements added at the expense of starch.
[2] Average starting weight, 100 g; mortality was less than 5% of total.
[3] Purina Guinea Pig Chow, Ralston Purina Company, St. Louis.
[4] Average starting weights, 106 to 110 g; number of animals at end of experiment given in parentheses.

an equivalent amount of alcohol extract of alfalfa or of the alcohol residue was not effective, but a combination of the 2 fractions was not tested.

In the last half of 1962, only insignificant growth responses were obtained with alfalfa in some experiments, even when they were continued an extra week. The reason for this was unknown but was thought to be due to the presence of impurities or sparing factors in the basal ration, as well as to the loss of a possible labile factor in the alfalfa sample. Therefore, the basal diet was changed to GPD3 to improve the test. Results with this more simplified diet are listed in table 3. Animals fed the basal diet (GPD3) had a higher mortality rate than those fed the former diet. Significant growth responses were obtained with the 2 samples of dried alfalfa (the original sample and a new supply which has been used since, indicating that the factor(s) responsible for the increase in growth was reasonably stable when stored at cold temperatures for long periods). The separate additions of 0.2% inositol and 5 or 15% of cellophane [10] (ingredients in GPD2) were without effect. When an additional 1% of mineral mixture in combination with 3% of cellophane (simulating the mineral and cellulose content of 10% of alfalfa) was fed, there was no stimulation of growth, nor was there any effect of adding alfalfa ash equivalent to 10% of alfalfa. The crude grass juice fraction obtained from alfalfa gave a small but statistically significant response. (Whether the factor(s) is partially destroyed by the small amount of heat necessary to make the concentrate or whether a factor(s) present in the extracted residue must be present to realize the full growth-stimulating effect cannot be answered at this stage.)

A number of different foodstuffs and miscellaneous ingredients were tested for their growth-promoting activities before 1963, using the 2-weeks' test. Good responses, equivalent to alfalfa, were obtained in one or more experiments with a number of feedstuffs from plant sources (level fed given in parentheses): soybean meal (10%), dried broccoli (10%), dried grass clippings (10%), and stock diet (20%). Moderate growth responses were observed with dried kale (10%), corn meal (10%), whey (dried spray) (10%), corn cob flour (10%), distillers solubles (2%), and wheat bran (10%). Insignificant responses were obtained with lactose (10%), liver residue (3%), liver powder (3%), fish meal (3%), pinitol (0.2%) (14), inositol (0.2%), starch (20%), cellulose (15%), oxalic acid (0.2%), levulinic acid (0.1%), furfuraldehyde (0.2%), carotene (0.2%), a commerical

[10] Cellophane Spangles, Rayon Processing Company, Pawtucket, Rhode Island.

TABLE 3

Supplementation of diet GPD3

	Additions to diet GPD3 [1]	No. of guinea pigs	No. of experiments	Gain in 3 weeks [3]	Gain over basal
				g	*g*
1	None	62(51)	9	72 ± 5.2 [4]	—
2	10% dried alfalfa, sample 1	17(15)	2	112 ± 5.3	+40
3	10% dried alfalfa, sample 2	40(36)	6	122 ± 3.8	+50
4	0.2% inositol	7(5)	1	72 ± 11.8	—
5	5% cellulose [5]	6(6)	1	88 ± 9.1	+16
6	15% cellulose	7(6)	1	74 ± 9.0	+ 2
7	1% HMW salts [6] + 3% cellulose	16(12)	2	70 ± 4.6	− 2
8	1.8% alfalfa ash	10(9)	2	65 ± 6.2	− 7
9	Alfalfa juice concentrate (equivalent to 10% alfalfa)	34(27)	5	95 ± 7.7	+23
10	Stock diet [7]	38(35)	5	118 ± 4.9	+46

[1] Supplement added at the expense of sucrose.
[2] Number of animals left at end of experiment given in parentheses.
[3] Average starting weights, 102 to 106 g.
[4] Mean ± SE.
[5] Cellophane Spangles, Rayon Processing Company, Pawtucket, Rhode Island.
[6] See table 1, footnote 3.
[7] Purina Guinea Pig Chow, Ralston Purina Company, St. Louis.

source of an unidentified factor used in poultry rations (0.3%), citrus tannins (0.3%), saponin (0.1%), Torula yeast (5%), brewer's yeast (5%), xylose (3%), a variety of flavoring agents (0.3%), additional amounts of all vitamins, and various mixtures of amino acids, including arginine and methionine alone or in combination. Two acetone-soluble fractions of alfalfa juice concentrate [11] were inactive. A combination of 0.1% inositol, 15% cellulose, and 20% starch (as in GPD2) gave a small but definite growth response that none of the components gave when fed alone.

DISCUSSION

The studies of Reid (10) on vitamin requirements of the guinea pig have resulted in improved knowledge of their nutrient requirements. However, little information is known about possible unidentified factors for guinea pigs. The review of Mannering (15) was published before the above review by Reid, and it is now evident that diets used to test former "unidentified factors" were low in either known vitamins, such as folic acid or thiamine, or in minerals, such as potassium, magnesium, or zinc. In the present studies, the addition of 10% of dehydrated alfalfa to the guinea pig's semipurified diet invariably resulted in better growth than that obtained when guinea pigs were fed alfalfa-free diets containing what is believed to be adequate amounts of all known vitamins, minerals, and amino acids.

These studies indicate that the reduced growth of young guinea pigs is due to the absence of some unidentified growth factor(s). Since the supplementation with alfalfa meal and many other ingredients of plant origin gave a significant growth response, it appears that the unknown growth factor(s) is widely distributed in plant sources. The animal products tested were either inactive or had low growth-promoting activity. The relationship of this "plant factor(s)" to the "grass juice factor(s)" of Kohler and co-workers (2, 9) or to other reported unidentified factors found in alfalfa or similar plant substances cannot be postulated at this stage. Also, we do not know whether the unidentified factor(s) acts by suppressing low-grade infections, but this is a possibility. Various combinations of antibiotics fed at different levels have not given similar results. Studies are in progress, in cooperation with Kohler and his co-workers at the USDA Western Regional Research Laboratory, in attempts to define the properties and nature of this unidentified factor(s).

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